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**Sustained Regeneration of High-volume Adipose Tissue for Breast
Reconstruction using Computer Aided Design and Biomanufacturing**

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Abstract

Adipose tissue engineering offers a promising alternative to the current breast reconstruction options. Here we investigated patient-specific breast scaffolds fabricated from poly(D,L)-lactide polymer with pore sizes >1 mm for their potential in long-term sustained regeneration of high volume adipose tissue. An optimized scaffold geometry was modelled *in silico* via a laser scanning data set from a patient which underwent breast reconstruction surgery. After the design process scaffolds were fabricated using an additive manufacturing technology termed fused deposition modelling. Breast-shaped scaffolds were seeded with human umbilical cord perivascular cells and cultured under static conditions for 4 weeks and subsequently 2 weeks in a biaxial rotating bioreactor. These *in vitro* engineered constructs were then seeded with human umbilical vein endothelial cells and implanted subcutaneously into athymic nude rats for 24 weeks. Angiogenesis and adipose tissue formation were observed throughout all constructs at all timepoints. The percentage of adipose tissue compared to overall tissue area increased from 37.17% to 62.30% between week 5 and week 15 ($p < 0.01$), and increased to 81.2% at week 24 ($p < 0.01$), while the seeded endothelial cells self organised to form a functional capillary network. The presented approach of fabricating customised scaffolds using 3D scans represents a facile approach towards engineering clinically relevant volumes of adipose tissue for breast reconstruction.

Keywords:

Biofabrication, scaffolds, adipose tissue engineering, breast reconstruction, , CAD/CAM, 3D imaging

1.0 Introduction

Breast cancer is a global problem and a major cause of morbidity, with an estimated number of 300,000 new cases diagnosed in 2013 [1]. While treatment concepts have moved from radical mastectomy to lumpectomy and adjuvant therapies such as chemotherapy, radiation or hormone therapy, there is still a significant number of patients suffering from the consequences of surgical removal of breast tissue. Such procedures often have negative psychological effects on the well-being of the patients. Earlier studies by Renneker *et al.* [2] showed that mastectomy is directly related to a psychological syndrome “marked by anxiety, insomnia, depressive attitudes, occasional ideas of suicide, and feelings of shame and worthlessness” [2]. Although several new approaches exist today in order to anatomically reconstruct the breast, some women still report the feeling of an altered body image [3]. This might partly be due to the fact that reconstructive methods available today repair, yet do not regenerate native adipose tissue, which is necessary for restoring the natural shape and feel of a breast. Prosthetic silicone-based implants are associated with a high occurrence of a mid and long-term foreign body response in form of a fibrous implant encapsulations altering the shape of the breast and more importantly leading to patient pain and discomfort [4-6]. Autologous fat tissue transplantation and free tissue flap transfers are also often used by plastic surgeons. However are associated with a high risk of tissue shrinkage, fat necrosis and/or oil cyst formation [7, 8].

To overcome the limitations of the current breast reconstructive techniques, impetus has been steadily growing towards cell-based regeneration of adipose tissue. Tissue Engineering and Regenerative Medicine (TE&RM) approaches aim to move the field away from methods to replace damaged tissue with permanent implants to more

biological solutions that are able to restore structure and function of autologous tissue [9]. Since the publication of the pioneering research paper in year 1999 by Patrick *et al.* [10] who used preadipocyte-seeded polyglycolic acid (PLGA) scaffolds for regenerating small volumes of adipose tissue, research groups from around the world [11-19] have achieved breakthroughs which moved the field forward. However, formation of sufficient volumes of mature adipose tissue remains a major problem and so far it has not been possible to maintain the structural entity of tissue engineered adipose constructs for more than 8-16 weeks [20, 21]. Current developmental efforts are therefore directed towards resolving problems that prevent the upscale of adipose tissue engineering concepts towards clinically relevant volumes that can be maintained throughout the life of the patient.

The shape and size of the breast of each patient is different, hence the scaffold of each breast reconstruction should be customized [9, 22, 23]. Previous studies undertaken in our laboratory have established a clinically viable methodology to design scaffolds for breast tissue engineering applications from patient scan data sets [24]. In this project, our main focus was on the design and fabrication of anatomically-shaped and patient-specific scaffolds using a biopolymer that degrades over the time scale of 1-2 years. Previous reports have indicated that a scaffold made out of such a slow-degrading polymer provides a long-term stable platform for the adipose tissue to regenerate and to mature, remodel its environment and stabilise [25]. It is well known that not only large but fully interconnected pore architectures facilitate cell invasion and blood vessel ingrowth crucial for maintaining long-term viability of adipose tissue [26, 27]. Since the diameters of a great number of the larger rat blood vessels infiltrating into a tissue-engineered construct lie in the 500-1500µm range [28], we hypothesized that a slow-degrading biopolymer with

pore sizes in the range 1000-2000 microns allows for efficient vascular in-growth and long-term sustained regeneration of high volume adipose tissue within a rat subcutaneous model.

2.0 Methods and Materials

2.1 Image acquisition and Computer Aided Design (CAD) model generation

A 3D CAD file was generated based on a 3D laser scan performed on a 46 year old female patient who suffered from an invasive ductal carcinoma from a data set previously derived [24]. Briefly, a Vivid 910 dot-laser scanner with built-in high precision camera (Konica Minolta, Marinouchi, Japan) was used to perform a 3D scan of the patient from three angles (0° frontal, and -30° and +30° oblique anterior). The images were imported in Rapidform2006 (Inus Technology, Seoul, South Korea) and merged into a single-shell object [29]. A custom surfacing algorithm was generated to model a virtual chest wall which was merged with the breast surface shell to obtain a watertight model of the solid breast. This watertight model was then meshed and exported as a Standard-Tessellation-Language (STL) file.

2.2 Design & Fabrication of scaffolds

The volume of the original CAD model (194 cm³) was scaled down to 3 cm³. Skeinforge-55, open-source slicing software, was used to generate machining computer-numerical-control (CNC) code from the CAD file. Custom slicing software was designed to selectively remove the perimeter shells of each layer. The resultant CNC code was then used to fabricate the scaffolds using the Replicator 3D printer (Makerbot Industries, New York, USA). During extrusion of the scaffolds, the layer thickness was set to 0.37 mm, porosity value was set to 90%, operating flow rate and feed rate were matched to 41 mm/s and extrusion temperature was set to 220°C.

2.3 MicroCT (μ CT) evaluation of scaffolds

The specimens were scanned on a Scanco μ CT40 (Scanco AG, Brüttisellen, Switzerland) at 8 μ m resolution, employing 55 kV and 145 μ A with 250 ms exposure time. Porosity, pore-size and filament-size distributions were obtained by employing a modified trabecular bone histomorphometry algorithm.

2.4 Isolation and culturing of primary human umbilical cord perivascular cells (HUCPVCs)

HUCPVCs represent a rich source of human mesenchymal cells found in the perivascular region of the human umbilical cord [30] and have recently gained attention in the tissue engineering field due to their short doubling time, and high occurrence of colony-forming-unit-fibroblast (CFU-F). HUCPVCs isolated from consenting full-term caesarean section patients were obtained from Tissue Regeneration Therapeutics Inc. (Toronto, Canada). The HUCPVCs were isolated as reported previously [30] and were received in passage 2. HUCPVCs were maintained in Dulbecco's Modified Eagle Medium (containing 4600 mg/L glucose) (Invitrogen, Carlsbad, USA) growth media supplemented with 10% (v/v) fetal bovine serum (Lonza, Basel, Switzerland) and 1% (v/v) Penicillin (10,000 U/ml) – Streptomycin (10,000 μ g/ml) (Invitrogen, Carlsbad, USA) in a humidified incubator at 37°C and 5% (v/v) CO₂.

2.5 Culturing of primary human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in F12K medium (Invitrogen, Carlsbad, USA) supplemented with 0.1 mg/ml heparin (Sigma, St Louis, USA), 0.05 mg/ml Endothelial Cell Growth Supplement (Millipore, Billerica, USA), 10% (v/v) fetal bovine serum (Lonza) and 1% (v/v) Penicillin (10,000 U/ml) – Streptomycin (10,000 μ g/ml) (Invitrogen,

Carlsbad, USA), in a humidified incubator at 37°C and 5% (v/v) CO₂. HUVECs were used between passages 2 and 8.

2.6 Production of GFP Lentiviruses and transduction of HUVECs

HUVECs were labelled with enhanced green fluorescent protein (eGFP) using a pLenti CMV GFP Puro (658-5) plasmid (Addgene 17448) and packaged with plasmids pRSV-Rev, pMDLg/pRRE and pMD2.G (contains VSV.G gene) (Addgene 12253, 12251 and 12259). The pLenti CMV GFP Puro plasmid were amplified in *Escherichia coli* and purified using W/Endo-free Qiagen Maxi-Prep Kit (Promega) according to the manufacturer's instructions and packaging plasmids were amplified in *Escherichia coli* and purified using Maxi-Prep Kits (Qiagen). Packing cell line 293T cells (GenHunter Corp.) in a T75 flask were transfected with 6.6 mg pLenti CMV GFP Puro (empty) and 3.3 mg of each packaging plasmids pRSVRev, pMDLgpRRE and pMD.G in 133 µl 1.25 M CaCl₂, 0.5 ml H₂O and 0.66 ml 2 x HEPES buffered saline and the cells were incubated at 37°C/ 5% CO₂. Four hours later, the medium was removed, cells were washed twice with warm phosphate-buffered saline (PBS) and replaced with 5 mL of complete DMEM. The cell culture supernatant was harvested 48 hr later by centrifugation at 850 g for 7 min at 4°C, followed by filtration of the supernatant through a 0.45 µm filter. The viral supernatant was concentrated 80-100 times using Amicon Ultra 15 ml centrifugal filter units (Millipore). The lentiviral stocks were stored in small aliquot at -80°C for titration and cell transfection. Titer of the concentrated lentiviral was determined by plating 4x10⁵ 293T cells in 6-well plate and infecting the cells with serial (10-fold) dilutions of concentrated lentivirus. After 48 hours, cells were trypsinised, washed three times with cold PBS and fixed with 1% paraformaldehyde (PFA) for 30 mins. The fixed cells were analyzed using

FACSCalibur for eGFP expression and for a typical preparation, the titer was approximately 10^7 to 10^8 infectious units per ml. GFP

HUVECs (2×10^6) in T175 flask were infected with 10 infectious units per cell in 5 ml of complete DMEM with polybrene (8 $\mu\text{g/ml}$) (Santa Cruz biotechnology) for 1 hr incubation at 37°C / 5% CO_2 before the addition of 15 ml of complete DMEM/polybrene. Incubation was continued for 24 hrs before DMEM/polybrene media was replaced by complete DMEM. 48 hours post-transduction, the media was replaced with DMEM complete with 0.5 $\mu\text{g/mL}$ of puromycin (Invitrogen), as determined previously by a puromycin kill curve (0.2-4 $\mu\text{g/mL}$) with HUVECs, in order to select for eGFP positive cells.

2.7 Preparation of cell-seeded TECs

Breast-shaped PDLLA scaffolds were first washed with sterile PBS and for sterilisation immersed in 70% ethanol overnight. Once the ethanol evaporated, the scaffolds were subjected to UV-irradiation for 30 minutes on each surface. Sterilised scaffolds were later placed in PBS overnight to pre-wet them. HUCPVC were detached using 0.25% trypsin, washed with PBS and resuspended in culture media. 20×10^6 HUCPVC were seeded onto each scaffold within a fibrin matrix (Baxter, Toongabbie, Australia) to improve cell attachment. After trypsinisation, the cell suspension was diluted with thrombin solution at a ratio of 1:1. 600 μL of cell/thrombin suspension was mixed with 600 μL fibrinogen solution and seeded on each scaffold. Cell-seeded scaffolds were cultured at 37°C and 5% CO_2 in complete DMEM.

After 4 weeks of static culture, all scaffolds were subjected to a dynamic culture environment for 2 weeks utilising the TisXell biaxially rotating bioreactor (QuinXell

Technologies, Singapore) for 8 hours per day at 5 rpm. The bioreactor setup has been previously described elsewhere [31, 32].

2.8 *In vivo* implantation into athymic nude rats

The animal experiments were approved by the Animal Ethics Committee of the Queensland University of Technology (ethics approval number: 11-147). Three 7-weeks old male athymic nude rats were purchased from the Animal Resources Centre (Perth/Australia). Rats were anaesthetized with a mixture of Ketamine (100 mg/kg) and xylazine (10 mg/kg). Surgeries were performed in a laminar hood under sterile conditions. A single 3 cm midline incision was made over the spine of the rats. Subcutaneous pockets were created on both flanks by blunt dissection and one scaffold was placed in each pocket. Before the wound was closed, scaffolds were injected with a solution of HUVEC encapsulated in Matrigel (1.3×10^6 cells per scaffold). The wound was closed with clips and swabbed with sterile iodine. At the end of the surgery, analgesic (Buprenorphine at 0.05 mg/kg s.c.) was administered subcutaneously.

2.9 Fluorescent imaging

In vivo imaging to detect the GFP signal from HUVEC was performed on the IVIS Spectrum imaging system (Caliper Life Sciences, Alameda, California) every 3 weeks. Rats were anaesthetized with 5% isoflurane and remaining fur was gently removed. Imaging was performed by setting the emission filter at 540 nm, excitation filter at 500 nm, exposure time at 0.2 seconds and luminescent exposure at 2 seconds. The images were imported into IVIS Living Image (version 4.3.1) software (Caliper Life Sciences, San Jose, USA) and native tissue autofluorescence was subtracted using a spectral unmixing algorithm. Once the autofluorescence was subtracted, all images were normalised to a minimum colour scale of 800 counts.

2.10 Mechanical Testing

Compression testing was performed on the explanted TECs using an Instron 5848 microtester fitted with a 500 N load cell (Instron, Norwood, USA). Since the top and bottom surfaces of the breast scaffolds are convex in nature, a support and a loading mould (illustrated in Supplementary Fig 3) were fabricated in order to evenly distribute the forces on the TECs. The supporting structures were fabricated with Acrylonitrile butadiene styrene (ABS) polymer with a young's modulus of 3.5 GPa. In order to minimise mechanical damage to the soft tissue, the testing protocol comprised a 2 mm compression of the scaffold at a rate of 0.6 mm/min.

2.11 μ CT Angiography

Scaffolds (n=3) were used for μ CT angiography. All animals were euthanized by CO₂ asphyxiation 6 months post implantation. Radiopaque contrast agent-enhanced μ CT angiography was performed using a protocol adapted from Duvall *et al.* [33]. Briefly, a surgical catheter was inserted into the left ventricle of the heart and advanced into the ascending aorta. Isotonic saline was perfused into the vascular system using a peristaltic pump until the blood was washed out. Following this procedure, a polymerizable, lead chromate-based, radiopaque contrast agent (Microfil MV-122, Flow Tech, Carver, USA) prepared according to manufacturer's instructions was perfused into the vascular system. Animal carcasses were stored at 4°C for 24 hours to allow polymerisation of the contrast agent. Scaffolds were then removed from the animals and stored in 4% PFA for 24 hours until μ CT analysis.

2.12 Histological and immunohistochemical analyses

2.12.1 Hematoxylin & Eosin (H & E)

Tissue was harvested from the rats after 5 and 15 weeks using a 5 mm biopsy punch penetrating through the entire height of the TECs (see Supplementary Fig 5). After explantation, TECs were fixed with 4% PFA, dehydrated and embedded in paraffin using a tissue processor (Excelsior ES, Thermo Scientific, Waltham, USA). Constructs were horizontally sliced to 5µm, deparaffinised with Xylene, rehydrated with a decreasing series of ethanol and stained with H & E. Stained slides were scanned with the SCN400 slide scanner (Leica, Solms, Germany) at 20x magnification.

2.12.2 Immunohistochemical staining

Endogenous peroxidase activity was blocked by incubating the slides with 3% H₂O₂ at room temperature. Slides were washed three times with Tris-HCL (pH 7.4) for 2 minutes and placed in tri-sodium citrate buffer for 20 min at 95 °C in a decloaking chamber. The slides were incubated with 2% bovine serum albumin (BSA) for 60 minutes at room temperature and incubated with prediluted corresponding primary antibody overnight at 4°C. All the four primary antibodies used are listed in Supplementary Table 1. The slides were then incubated with immunoglobulin as secondary antibody as part of the DAKO Envision Dual Link System-HRP (Ref: K4061, Dako, Glostrup, Denmark) for 30-45 min at room temperature. The slides were washed 3 times with Tris-HCl and colour was developed by incubating the slides with 3,3'-Diaminobenzidine (DAB) solution (1:50) [DAKO liquid DAB+ substrate Chromagen System, Ref: K3468, Dako, Glostrup, Denmark]. Finally, the slides were washed once with Tris-HCl for 2 minutes and counter-stained with Haematoxylin for 2 minutes followed by 30 second immersion in 0.1% Ammonium Hydroxide.

2.13 Histomorphometry

Histomorphometrical analyses were undertaken using two methods: a manual method and an automated approach. The manual method was undertaken using the Osteomeasure histomorphometry analysis system (Osteometrics Inc., Decatur, GA, USA). All measurements were performed blinded on 4 consecutive sections from each scaffold at each time point. To determine the average adipose tissue area, the total area of the adipose tissue was first calculated (A). Secondly, the total area occupied by the scaffold struts was measured (S). Finally, the combined area of the tissue section was measured (C). The ratio of adipose tissue area to total tissue area (R) was calculated using the following formula:

$$R = \frac{A}{(C - S)} * 100\%$$

ImageJ (National Institutes of Health, MA, USA), in conjunction with Adipocyte Tools plugin developed by Montpellier RIO Imaging (Montpellier, France), was used for all automated calculations involving cell size distribution. The field of view (FOV) from each histological section was kept uniform at 4795 x 2282 µm. Background was first removed from each histological section by the pre-processing macro within the Adipocyte Tools plugin using the thresholding method. Minimum size of each cell was chosen to be 40 µm, maximum size as 800 µm and the number of dilates were set to be 10. The same threshold was also chosen to automatically set regions of interest (ROI) around the adipose cells. The automated method generated a small number of ROI artefacts. Artefacts that could be detected visually were manually removed. In order to remove the remaining artefacts, 10% of the smallest and 10% of the largest ROIs were excluded from any further analysis. Visualisation of both the manual as well as the automatic method can be found in Supplementary Fig 2.

Fibrotic capsules were identified from H & E stained histological sections as a dense layer of collagen fibres aligned parallel to the implant surface with a variable presence of fibroblasts and inflammatory cells [34]. 3 images chosen randomly from each scaffold were imported in ImageJ and measurement lines, approximately 50 μm apart, were defined on the fibrotic capsule. Average thickness of the fibrotic capsule was derived from the average of such measurement lines.

2.14 Statistical analysis

All data are represented as mean \pm SD and are subjected to one-way analyses of variance (one-way ANOVA) and Tukey's post-hoc test (Sigmaplot 12.5). Significance levels were set at $p < 0.05$. All error bars represent standard deviation.

3.0 Results

3.1 Characterisation of scaffolds

The gross morphology of the fabricated PDLLA scaffolds was highly similar to the CAD file obtained from the laser scanning of the breast region (Fig 1. a-c) and contained a regular array of interconnected pores. The axial pores of the scaffolds were larger than the transverse pores of the scaffolds. This observation was confirmed by μCT measurements (Fig 1(e)), where the smaller pore sizes (200-400 microns) reflected the transverse pores of the constructed scaffolds as seen in Fig 1(d, right). The larger pore sizes (500-1000 microns) reflect the axial pores of the scaffolds as seen in Fig 1(d, left). The high porosity value of the scaffolds also implies more volume available for tissue/vascular ingrowth. From the 3D μCT reconstructed images it was also shown that the filament thicknesses across the entire scaffolds were largely homogeneous (Fig 1d).

3.2 *In vitro* culture

To improve the seeding efficiency of cells onto the large volume scaffolds, a clinically approved hydrogel system, namely fibrin glue, was used. A fibrinogen/cell suspension was mixed with thrombin and immediately added to the scaffolds so that the fibrin clot was formed *in situ* within the scaffold pores. The scaffolds were also pre-wet in sterile PBS overnight prior to seeding them with cells. Initially, when the cells were encapsulated in fibrin glue, they showed a rounded morphology, however as shown in the light microscopy image in Supplementary Fig 1a, after 24 hours, filopodia began to develop. After day 28 days of static culture cells were seen to be bridging across the pores of the scaffold (Supplementary Fig 1b). The rotational motion of the TisXell bioreactor leading to increased perfusion of nutrients facilitated cell growth, as observed from the size and number of bridges across the pores, which increased significantly in 2 weeks under dynamic culture (Supplementary Fig 1b, c).

3.3 Fluorescent imaging

Prior to the implantation of scaffolds into rats, the pre-cultured scaffold/cell constructs were further seeded with a suspension of GFP-labelled HUVECs in Matrigel in order to enhance the angiogenic potential of the TECs. The rats were subjected to *in vivo* fluorescence imaging every 3 weeks to detect the fluorescent signal from the implanted GFP-labelled HUVECs. The fluorescence signal could not be distinguished from the background noise on week 0. On week 2, the signal was localised at the right wing of the scaffold (Fig 2a). Over the next 15 weeks, the signal persisted at the initial location and seemed to invade towards the central regions of the scaffold (Fig 2 b-f).

3.4 Scaffold explantation and degradation

No postoperative infections or macroscopic signs of foreign body reaction to the TECs occurred over the entire period of implantation. After 6 months of implantation, the TECs were retrieved for histological analysis. Upon visual examination, it was shown that the scaffolds were well integrated into the host body with no apparent major inflammatory reaction (Fig 3 a). Visual examination of the Microfil perfused samples revealed a widespread invasion of host vasculature into the engineered adipose tissue (Fig 3 b, c, d). MicroCT angiography of the scaffolds revealed a high degree of vascularisation equally distributed throughout the architecture (Fig 3c). Furthermore, it was also found that the scaffolds did show, as anticipated, significant degree in molecular weight loss (data not shown) (Fig 3 e). Our tissue engineering concepts [35] are based on the principle that the overall shape of the scaffold remained intact (Fig 3d).

3.5 Formation of vascularised adipose tissue

Hematoxylin & Eosin staining revealed formation of adipose tissue and blood vessels across all time points (Fig 4 a-h). H&E staining of tissue samples explanted at week 5 (Fig 4 a & b) and 15 (Fig 4 c & d) showed a well vascularised adipose tissue construct. H&E staining of tissue samples explanted at week 24 demonstrated widespread regeneration of well-vascularised adipose tissue throughout the length, width and height of the scaffolds. No regions of necrosis, inflammation or cysts were observed.

All constructs showed the formation of a thin fibrotic capsule around the constructs. Adipose cells could be identified microscopically by their typical ring-like morphology and a large vacuole in the middle of the cell. These characteristics enabled quantification of the area of fat tissue.

The percentage of adipose tissue compared to overall tissue area increased from 37.17% to 62.30% between week 5 and week 15, and grew to 81.2% between week 15 and week 24 (Fig 5a). The stiffness of the TECs decreased significantly from 3 MPa to 1 MPa over 24 weeks of *in vivo* implantation (Fig 5b). The results from the fibrotic capsule thickness measurements are displayed in Fig 5c. The semi-quantitative analysis of the thickness revealed that the size of the capsule increased from approximately 350 μm to 450 μm between week 5 and 15. However, as the scaffold degraded over time, the size of the capsule decreased to 200 μm as observed at week 24.

Quantification of adipose cell area allowed the visualization of the distribution of different-sized cells as a histogram (Fig 5d). At week 5, the majority of the cells had a surface area between 200 and 500 μm with the histogram skewing towards the right and a small number of outliers. At week 15, the number of cells counted increased considerably, but the histogram continued to remain skewed to the right with a higher number of outliers compared to those at week 5. The majority of cells had a surface area between 100 and 300 μm at week 15. The histogram at week 24 was more balanced compared to week 5 and 15, however it had the highest spread of values, having a considerably higher number of cells with a surface area larger than 1000 μm . The cell count also increased significantly between week 5 and 15.

Immunohistochemical staining for anti-human-mitochondria antibodies showed that a majority of the adipose tissue within the scaffold was of host origin, with human adipocytes aggregating into a small number of islands near the scaffold strands (Fig 6 h , i). Staining for anti-GFP showed that a significant number of HUVECs self-organised into a functional capillary network within the scaffold strands (Fig 6 e , f). A majority of the capillaries formed by HUVECs had a diameter less than 100 μm , were

clustered into domains approximately 800 μm x 400 μm in size and localised on the periphery of the TECs. All such capillaries also had presence of red blood cells, indicating their functional nature. Anti-CD68 staining showed that the scaffolds invoked an unspecific inflammatory response in close proximity to the scaffold strands (Fig 6 b , c). CD68-positive macrophages were not found more than 400 μm away from the nearest scaffold strand showing that unspecific immune reaction was not widespread and localised within a radius of approximately 400 μm of the scaffold strands.

4.0 Discussion

The overall goal of breast reconstruction after mastectomy is to restore the patient's breast with functional tissue interfaces, and to maintain tactile sensation. The shape and size of the breast is different for each patient, hence the scaffold supporting the regeneration of the tissue interfaces needs to be customised. Here, the translation of scaffold-based tissue engineering applying the toolbox provided by Computer-Aided-Design and Computer-Aided-Manufacturing (CAD/CAM) opens up new vistas for scaffold-based breast reconstruction. In this article, we present an integrated strategy where images are first taken of the breast region of a mastectomy patient using medical imaging techniques. The images captured can then be processed into a 3D computer-aided design (CAD) model which is then sent to a bioprinter to be fabricated into a highly porous biodegradable scaffold. The porosity and pore sizes can be tailored independently by changing the laydown pattern. The optimal porosity is always determined by a trade-off between mechanical properties and pore volume available for tissue ingrowth [36]. Stiffness and strength should be sufficient in the context of breast tissue engineering, as the scaffold should be sufficiently robust to not only resist changes in shape *in vitro* as a result of cell contraction forces yet also

the wound contraction forces which will be invoked during tissue regeneration. Furthermore, the breast region is also subjected to high biomechanical loads during sleeping and sports activities, and the TECs must be able to withstand those forces over a long period of time.

Patrick *et al.* were among the first to study adipose tissue engineering *in vivo* [10]. Since then several research groups have developed different approaches to engineer fat tissue using different types of cells and/or scaffolds. Most of these studies used small volume scaffolds and implants (Supplementary Fig 4); therefore, there is a direct need of sustained regeneration of clinically relevant high-volume adipose tissue. Furthermore, very few studies have analysed the long-term behaviour of tissue engineered breast constructs to date. To our knowledge, we present the first such study utilising a large-volume patient-specific TEC in an *in vivo* model and achieving a sustained regeneration of adipose tissue up to 24 weeks.

The volume of the originally designed scaffold (194 cm^3) was scaled down for the rodent experiment to 3 cm^3 (Table 3); yet it is important to note that it is a considerably larger volume than reported in most previous studies (for a comparison of volumes of scaffolds used for adipose tissue engineering, please see Supplementary Fig 4) was reconstructed. Furthermore, a 3 cm^3 scaffold leads to a breast/body volume of 0.84% in a rodent, considerably larger than that of an average human female (0.63%). For a detailed comparison, see Supplementary Table 2.

It is important to note here that, based on our tissue engineering philosophy [35, 37] we did not aim to produce a scaffold whose mechanical properties matched those of native adipose tissue. PDLLA has been also been used previously to engineer adipose tissue [38, 39]. PDLLA is an aliphatic polyester which is characterized by bulk

degradation and has a modulus of 1.5 GPa [40, 41]. Our hypothesis was that the highly porous scaffold would maintain its shape and volume for at least 9-12 month without showing any signs of mass loss so that the regenerated tissue stably remodels several times in the highly porous scaffold architecture without being influenced by any degradation by products.

As the scaffolds showed loss of molecular weight in both the *in vitro* and *in vivo* phase (data not shown), their mechanical properties also changed significantly over a period of 8 month (2 month *in vitro* and 6 month *in vivo*). Measurement of the Young's modulus after explantation revealed that the TECs had 33% of the Young's modulus compared to the original scaffold values; however, the form stability and integrity and form specificity remained unchanged, with the TEC recovering shape and volume completely after being subjected to 10% compression. The stiffness of the scaffold was measured at 3 MPa in comparison to the TEC which had a stiffness of 1 MPa post-implantation (Fig 5b) which is 2 orders of magnitude stiffer compared to native adipose tissue. As PDLLA degradation occurs throughout the whole via bulk degradation the scaffold did lose its mechanical strength uniformly throughout its volume [42]. However, as per our hypothesis, the biodegradable scaffold was able to withstand the contraction forces for at least 6 months *in vivo* and did not show any mass loss or volume contraction.

Scaffold architecture has a major impact on adipose tissue formation. Scaffolds morphologies formed by Fused Deposition Modelling (FDM) are homogeneously organised with precisely deposited struts and contain a highly interconnected channel network. Previous work within our group [43] and elsewhere [21] have shown that scaffolds containing small pores and pore interconnections are unsuitable for high volume adipose tissue regeneration. Diffusion of oxygen from the

surrounding tissues is sufficient only over a distance up to approximately 150 μm [44]. Therefore, in tissue constructs without adequate vascularization, autologous adipose tissue loses up to 60% of its volume post-transplantation because the cells in the centre of the graft are inadequately supplied with nutrients and transport of waste is not possible [45, 46]. Pore sizes of 1.5 mm used in the presented study allowed vascular ingrowth into the scaffold during the early stages of the implantation which helped sustain the adipose volume for an extended period of time.

Our results from anti-CD68 staining show the presence of macrophages, primarily bordering the scaffold strands which can be interpreted as an unspecific immune reaction. The invasion of these macrophages may have had a large role to play in the subsequent adipogenesis and vasculogenesis. Moldovan [47] and Anghelina *et al.* [48, 49] have shown that upon implantation, monocytes and macrophages first invade the extracellular matrix around the graft and form tunnels while clearing ECM along their path. Subsequently, endothelial cells and adipocytes are visualised in these macrophage-lined tunnels, indicating their development into functional capillary-like structures and vascularised adipose tissue. However, further investigations done by Debels *et al.* [50] have found no conclusive evidence of macrophages playing a direct role in neoadipogenesis and claim that the perceived neoadipogenesis is simply a result of increased angiogenesis, without further involvement of macrophages.

Immunohistochemistry on paraffin embedded explants samples with human-specific mitochondria antibody confirmed the host derivation of the newly formed adipose tissue, indicating that the regeneration process is dependent on inductive factors embedded within Matrigel or excreted by the transplanted cells, rather than direct tissue formation by provided precursor cells. This finding complies with a study

performed by Stillaert *et al.* [51] who also used Matrigel in combination with FGF2 and concluded that such an inductive graft causes the recruitment and differentiation of host-derived precursor cells into adipose cells. Previous studies [52, 53] have shown that adult adipose tissue houses a significant number of mesenchymal stem cells with pluripotential characteristics. There is a high likelihood that these cells were significantly involved in the neoadipogenesis process. Athymic nude rats are characterised by their lack of normal thymus and functionally mature T cells and are therefore useful in xenograft models [54]. However, they still possess allo- and xeno-reactive natural killer cells that can recognise and kill major histocompatibility complex (MHC) incompatible cells through the recognition of both mismatches in the classical (RT 1.A) and non-classical (RT1.C/E) MHC class 1 regions. With increasing age, nude rats develop leukocytes, for example dendritic cells, T-like cells and T-cell receptors which may also contribute to specific innate immune responses [54]. At the time of implantation, it is reasonable to assume that the HUCPVC were mostly localised on the scaffold strands, which is also the region encapsulated by fibrous tissue and granulomatose reaction. It is therefore likely that the implanted HUCPVC were constantly triggering a response by the innate immune system of the host and did not survive long-term.

As described previously, one of the goals of this research project was to construct a human vascular bed within a nude rat model. The aims of such a pursuit are not limited to tissue engineering applications where vasculogenesis by means of precursor cell induction can provide nutrition to adipose cells, but also to basic cancer research whereby humanised vascular structures can be used as a model to study the growth and proliferation of cancer metastases [55]. *In situ* fluorescent imaging showed that on week 2, the eGFP signal from the implanted HUVECs was

localised onto to the right wing of the scaffold. This observation was unexpected since the HUVECs were distributed homogeneously throughout the scaffold. One argument is that the movement of the rat and gravitational forces caused the cells to accumulate on the bottom region of the scaffold. Another possibility is that the HUVECs in the core regions of the scaffolds perished due to hypoxia, while those on the periphery survived owing to a greater nutrient exchange at these sites.

Anti-GFP staining showed that in contrast to HUCPVC, GFP-labelled HUVECs survived the 6-month implantation process all throughout the scaffolds. However, their self-assembly into capillaries of diameters less than 100 μm was observed only around the periphery of the scaffold. The clustering of cells in the posterior regions of the scaffold may have led to increased cell-to-cell contact in these regions, which is a very important factor determining the extent of capillary network formation with a minimum critical density required for optimal capillary bed formation [56, 57].

Although further investigation is needed, the maintenance of such a humanised capillary bed in a nude rat model for 24 weeks *in vivo* opens up the possibility of generating vascular networks spanning a greater area for many tissue engineering approaches.

6.0 Conclusion

The development of a clinically translatable method of engineering breast adipose tissue requires investigation of several components. There must be coordination between all key aspects of the tissue engineering process, including the selection of cell source, scaffold design and material, cellular environment and means of surgical strategy, in order for the engineering of breast tissue to be successful. To our knowledge this is the first study showing a sustained regeneration of high volume

adipose tissue over a long period of time using a customized biodegradable scaffold designed by utilizing 3D laser imaging data sets acquired from a breast reconstruction patient.

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Figure Captions:

Fig 1. Scaffold fabrication and characterisation. (a) Illustration of laser scanning performed on a patient suffering from invasive ductal carcinoma. (b) The data from the laser scan was used to generate a CAD model of the patient's left breast. (c) The CAD model was used to generate porous patient-specific scaffolds (shrunk down to 25% of their original size) showing high shape fidelity to the CAD model. (d) MicroCT scans of the fabricated scaffolds showed homogeneity of filament thickness across the entire volume of the scaffold. (e) Histogram of pore size measurements showing the distribution of pore sizes in the scaffolds. (f) Scaffolds fabricated with a highly reproducible porosity of 90 %. (g) SEM images showing the surface of the scaffold bars and struts.

Fig 2. Fluorescence signal from the GFP-labelled HUVECs detected using an IVIS bioluminescence scanner. Due to rapid movements of the rats immediately after the implantation of the scaffold, the HUVECs injected in the scaffold pockets of all rats got dislocated and accumulated in the posterior region of the scaffold. The signal from viable HUVECs increased steadily from week 2 until week 17.

Fig 3. (a) Scaffolds explanted after 24 weeks showed good integration with the host tissue with no observable signs of inflammation and fibrotic encapsulation. (b) Perfusing the animals with Microfil allowed the visualisation of all blood vessels within the scaffold and the surrounding tissue areas. The arrows in yellow show large blood vessels supplying oxygen and nutrients to the tissue within the scaffolds. (c) 3D image obtained from microCT reconstruction of the scaffolds explanted from Microfil-perfused animals showing a well-established network of capillaries throughout the entire volume of the scaffold. (d) Top view and (e) side view of fabricated scaffold (left) and explanted tissue engineered constructs (TECs) (right) after 24 weeks. The distribution of capillaries can be qualitatively visualised on the explanted TECs.

Fig 4. (a, b, c, d) Hematoxylin and Eosin (H&E) staining of tissue samples explanted at week 5 (a,b) and 15 (c,d) show a well vascularised adipose tissue and no signs of major inflammation or cyst formation. (e, f, g, h) H&E staining of samples explanted at Week 24. The scaffolds were sectioned in two orientations: transverse (e, g) and longitudinal (f, h). Representative sections are shown. Widespread regeneration of well-vascularised adipose tissue was observed throughout the length, breadth and height of the scaffolds. No regions of necrosis, major inflammation or cysts were observed.

Fig 5. (a) Box and whiskers plot showing the adipose tissue area relative to total tissue area over 24 weeks. Adipose tissue area increased significant ($p < 0.01$) from approximately 40% on Week 5 to approximately 60% on Week 15 finally reaching almost 80% on week 24 ($p < 0.01$). The variability across the samples was low, especially in the Week 24 tissue extracts. (b) Graph showing the compressive Young's modulus of scaffold pre- and post-implantation. Mechanical properties of the scaffold decreased significantly ($p < 0.01$) from 3MPa to 1MPa over 24 weeks *in vivo* implantation. The stiffness of native adipose tissue is represented by the red dashed line

[58] . (c) Box and whiskers plot showing mean thicknesses of the fibrous capsule surrounding the scaffold. As the scaffold degraded the thickness of the capsule decreased significantly ($p < 0.01$) from 400 μm to 300 μm between weeks 5 and 15 and down to 200 μm in week 24. The variability in the samples was high on week 15. (d) Histogram showing the distribution of adipose cells according to the cell surface area. Generally, the number of adipose cells increased significantly from week 5 to 24. At week 5, a majority of the cells had a surface area between 200 and 500 μm^2 . On week 15, the cells generally shrunk in area, with the majority of cells having an area between 150 and 350 μm^2 . Finally on week 24, the cells once again expanded in area and the majority had surface area between 200 and 500 μm^2 .*** statistically significant, $p < 0.01$.

Fig 6. Histological staining of scaffolds explanted on week 24. (a) Hematoxylin and Eosin (H & E) staining of scaffolds showing overall tissue morphology. (b & c) CD68 staining showing the presence of macrophages near the scaffold strands. Density of macrophages was low in areas away from the scaffold strands. (d) Anti-vWF staining for the blood vessels showing a well vascularised tissue. (e & f) Anti-GFP staining for the presence of GFP-labelled HUVECs showing the assembly of HUVECs into functional capillaries. (g) isotype control. (h & i) Staining for human mitochondria showing the host-origin of the regenerated tissue. Human fat cells (black arrow) were localised in small islands near the scaffold strands. Scale bars = 100 μm .

Supplementary Tables:

Supplementary Table 1. List of primary antibodies.

<u>Primary Antibodies</u>	<u>Description</u>
Anti-Von Willebrand factor	Polyclonal Rabbit Anti-Human Von Willebrand Factor: Ref A 0082 (DAKO, Glostrup, Denmark)
Anti-CD68	Monoclonal Mouse Anti Rat CD68: Ref MCA341GA (AbD Serotec, Oxfordshire, UK)
Anti-GFP	Rabbit Polyclonal Anti-GFP antibody: Ref AB290 (Abcam, Cambridge, UK)
Anti-human mitochondria	Mouse monoclonal anti-human mitochondria antibody: Ref AB92824 (Abcam, Cambridge, UK)

Table 2. Comparison of breast/body volumes of humans and rodents.

Type of Tissue	Volume (cm ³)	Breast/Body Volume	Ref
Whole human body	66,400	0.63%	[59]
Human breast volume	420		[60]
Whole rat body	353	0.84%	[61]
Breast scaffold volume	3		

Supplementary Figure Captions:

Supplementary Fig 1. (a) Cell morphology on day 1 post seeding suspended in fibrin glue. Cells do not remain rounded and are seen to spread and connect with neighbouring cells. (b) 28 days post culture the cells begin to form wide bridges across the gaps of the scaffolds (dotted by yellow triangles). (c) DAPI-Phalloidin staining of cells at 28 days post-culture to visualise cells forming a continuous sheet along the scaffold strands. Blue (DAPI): cell nuclei, Red (Phalloidin): cell actin filament.

Supplementary Fig 2. (a-c) Images depicting the workflow of the automated algorithm to count the number of adipose cells on a histology section and also their cell surface areas. (d-f) Images depicting the workflow showing manual demarcation of adipose tissue areas using Osteomeasure software.

Supplementary Fig 3. Schematic diagram of the test setup used for compression testing of scaffolds. Due to the curved nature of the scaffold surfaces, special top and bottom surface attachments following the contours of the scaffolds were produced. The top attachment links with the Instron microtester plunger providing uniform stress across the entire scaffold. The attachments were produced with non-porous acrylonitrile butadiene styrene (ABS) with a considerable higher stiffness compared to the 90% porous PDLLA scaffolds, to minimise their influence on the load readings.

Supplementary Fig 4. Graph showing comparison of volumes of scaffolds used for adipose tissue engineering research. Volume of the scaffold used in the present study is dotted by a horizontal line showing that it is considerably higher than the volumes used in most other studies.

Supplementary Fig 5. Illustration showing the position of the samples collected using biopsy punch outs at weeks 5 and 15. Areas shaded in dark purple represent tissue excised by the biopsy punch.